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(54) Title: NOVEL LIPOLYTIC ENZYME

(57) Abstract

The present invention relates to novel lipolytic enzymes. More specifically the invention provides novel lipolytic enzymes having the properties of a lipase native to the strain *Fusarium culmorum* CBS 513.94, or has immunochemical properties identical or partially identical to those of a lipase native to the strain *Fusarium culmorum* CBS 513.94.

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NOVEL LIPOLYTIC ENZYME

TECHNICAL FIELD

The present invention relates to novel lipolytic enzymes. More specifically the invention provides novel lipolytic enzymes having the properties of a lipase native to the strain *Fusarium culmorum* CBS 513.94, or has immunochemical properties identical or partially identical to those of a lipase native to the strain *Fusarium culmorum* CBS 513.94.

BACKGROUND ART

Lipolytic enzymes find multiple industrial applications. Alkaline lipases are of particular interest for use in detergent compositions.

Alkaline lipases of microbial origin have been described, including lipases obtained from *Fusarium*. However, lipases obtained from *Fusarium culmorum* have never been disclosed.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel alkaline lipolytic enzymes (EC 3.1.1.3).

Accordingly, in its first aspect, the invention provides lipolytic enzymes having immunochemical properties identical or partially identical to those of a lipase obtained from the strain *Fusarium culmorum* CBS 513.94.

In its second aspect, the invention provides a process for the preparation of the lipolytic enzyme, which process comprises cultivation of a lipase producing strain of *Fusarium culmorum* in a suitable nutrient medium, containing

carbon and nitrogen sources and other inorganic salts, followed by recovery of the lipolytic enzyme.

In its third aspect, the invention provides a process for the preparation of a lipolytic enzyme according to any of claims 1-6, which process comprises isolating a DNA fragment encoding the lipolytic enzyme; combining the DNA fragment with an appropriate expression signal in an appropriate plasmid vector; introducing the plasmid vector into an appropriate host either as an autonomously replicating plasmid or integrated into the chromosome; cultivating the host organism under conditions leading to expression of the lipolytic enzyme; and recovering of the enzyme from the culture medium.

In further aspects, the invention provides detergent compositions, as well as a detergent additives, comprising the lipolytic enzyme of the invention.

Finally, the invention provides a biologically pure culture of the strain *Fusarium culmorum* CBS 513.94.

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides novel lipolytic enzymes having the properties of a lipase native to the strain *Fusarium culmorum* CBS 513.94.

The Microorganism

The invention provides lipolytic enzymes derived from a strain of the fungus *Fusarium culmorum*. *Fusarium culmorum* is a known species and strains of *Fusarium culmorum* have been deposited and are publicly available from depositary institutes, e.g. Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Germany, and American Type Culture Collection (ATCC), U.S.A.

In a preferred embodiment the invention provides a lipolytic enzyme derived from the strain *Fusarium culmorum* DSM 1094, *Fusarium culmorum* DSM *Fusarium culmorum* 62184, *Fusarium culmorum* DSM 62188, *Fusarium culmorum* DSM 62191, *Fusarium culmorum* DSM 62223, *Fusarium culmorum* ATCC 12656, *Fusarium culmorum* ATCC 15620, *Fusarium culmorum* ATCC 16430, *Fusarium*

culmorum ATCC 16551, *Fusarium culmorum* ATCC 26556, *Fusarium culmorum* ATCC 34910, *Fusarium culmorum* ATCC 34913, *Fusarium culmorum* ATCC 36017, *Fusarium culmorum* ATCC 36879, *Fusarium culmorum* ATCC 36881, *Fusarium culmorum* ATCC 36886, *Fusarium culmorum* ATCC 44417, *Fusarium culmorum* ATCC 46040, *Fusarium culmorum* ATCC 56088, *Fusarium culmorum* ATCC 56089, *Fusarium culmorum* ATCC 60275, *Fusarium culmorum* ATCC 60362, *Fusarium culmorum* ATCC 62214, *Fusarium culmorum* ATCC 62215, or *Fusarium culmorum* ATCC 64075, or a mutant or a variant thereof.

In its most preferred embodiment the invention provides a lipolytic enzyme derived from the strain *Fusarium culmorum* CBS 513.94, or a muant or a variant thereof. This strain has been deposited according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Centraalbureau Voor Schimmelcultures (CBS), Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands, on 25 October 1994.

In another aspect, the invention provides a biologically pure culture of the strain *Fusarium culmorum* CBS 513.94.

Physico-Chemical Properties

In preferred embodiments, the lipolytic enzyme of the invention may be characterized by having one or more of the following physico-chemical properties.

The enzyme has a pH optimum in the range of from about 7 to about pH 9, more specifically around pH 8, when determined at 30°C with tributyrine as substrate.

The enzyme has the following N-terminal amino acid sequence (cf. SEQ ID NO:1):

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

The enzyme has a molecular weight of 28.4 kDa, as determined by mass spectrometry.

Immunochemical properties

In another preferred embodiment, the lipolytic enzyme of the invention is characterized by having having immunochemical properties identical or partially identical (i.e. at least partially identical) to those of a lipase obtained from the strain *Fusarium culmorum* CBS 513.94.

The immunochemical properties can be determined by immunological cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to *I. M. Roitt*; Immunology, Gower Medical Publishing (1985) or *N. H. Axelsen*; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), chapters 5 and 14. The terms "immunochemical identity" (antigenic identity) and "partial immunochemical identity" (partial antigenic identity) are described in Axelsen, supra, chapters 5, 19 and 20, and in I. M. Roitt, supra, Chapter 6.

Monospecific antiserum for use in immunological tests can be raised, e.g. in rabbits, against the purified lipase of the invention, e.g. as described in Chapter 41 of *N. H. Axelsen, supra*, or Chapter 23 of *N. H. Axelsen et al.*, A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications (1973).

Preparation of the Lipolytic Enzyme

The lipolytic enzyme of the invention may be produced by cultivation of a strain of *Fusarium culmorum* in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the lipase. In a preferred embodiment, the lipase producing strain is the strain *Fusarium culmorum* CBS 513.94, or a mutant or a variant thereof.

The lipolytic enzyme may also be obtained by recombinant DNA-technology by methods known in the art *per se*, e.g. isolating a DNA fragment encoding the lipase, combining the DNA fragment with appropriate expression signal(s) in an appropriate vector, introducing the vector or parts thereof into an appropriate host, either as an autonomously replicating plasmid or integrated into the chromosome, cultivating the host organism under conditions leading to expression of the lipase, and recovering the lipase from the culture medium.

In preferred embodiments of the invention, the host organism is of bacterial origin, preferably a strain of *Escherichia coli*, or a strain of *Bacillus*, or a strain of *Streptomyces*, or of fungal origin, preferably a strain of *Aspergillus*, a strain of *Neurospora*, a strain of *Fusarium*, or a strain of *Trichoderma*, or a yeast cell, preferably a strain of *Saccharomyces*, or a strain of *Kluyveromyces*, or a strain of *Hansenula*, or a strain of *Pichia*.

After the cultivation, the lipolytic enzyme may be recovered and purified from the culture broth by conventional methods, such as hydrophobic chromatography, ion exchange chromatography or combinations thereof.

Lipolytic Activity

The lipolytic activity may be determined using tributyrine as substrate. This method is based on the hydrolysis of tributyrin by the enzyme, and the alkali consumption is registered as a function of time.

One Lipase Unit (LU) is defined as the amount of enzyme which, under standard conditions (i.e. at 30.0°C; pH 7.0; and tributyrine as substrate) liberates 1 µmol titratable butyric acid per minute. Gum Arabic is used as emulsifier.

A folder AF 95/5 describing this analytical method in more detail is available upon request to Novo Nordisk A/S, Denmark, which folder is hereby included by reference.

Detergent Compositions

The lipolytic enzyme of the invention may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and

di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes conventionally used in detergent compositions, such as an amylase, a cutinase, a protease, a cellulase, a peroxidiase, and/or an oxidase.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O.2SiO_2$)	2 - 6%
Zeolite (as $NaAlSiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%

Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaA1SiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaA1SiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%

Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaAlSiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%

Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O.2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaAlSiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%

Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-18} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluensulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%

Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzene-sulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaA1SiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%
Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaA1SiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%

Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The lipolytic enzyme of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the lipase may be added in an amount corresponding to 0.001-100 mg of lipase per liter of wash liquor.

EXAMPLES

The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

Example 1

Cultivation Example

Seed cultures of the strain *Fusarium culmorum* CBS 513.94 were produced in 500 ml shakeflasks containing 100 ml of the following composition :

Corn steep liquer (dried)	12 g/l
Glucose	24 g/l

To each flask is added 0.5 g $CaCO_3$ and 0.5 ml of oil.
pH is adjusted to 5.5 before autoclavation.

After 3 days at 26°C and 250 rpm, 5 ml of each of the seed cultures were inoculated in shakeflasks containing 100ml of the following medium:

Pepton, Difco 0118	6 g/l
Pepticase, Sheffield Products	4 g/l
Yeast extract, Difco 0127	3 g/l
Meat extract, Difco 0126	1.5 g/l
Dextrose, Roquette 101-0441	1 g/l
Olive oil, Sigma	10 g/l

pH is adjusted to 7.3-7.4 before autoclavation.

Cultivation took place for 9 days at 26°C and 250 rpm. The broths were centrifuged and the supernatants purified on a hydrophobic matrix (TSK gel Butyl-

ToyoPearl 650 C column, available from Tosoh Corporation, Japan), and applied for further studies.

Example 2

Characterization Example

pH Optimum

The supernatant obtained according to Example 1 was subjected to the LU method for determining lipase activity described above, and the relation between pH and lipase activity of the lipolytic enzyme of the invention was determined at 30°C in the range of from pH 6 to pH 10.

The results of this characterization is presented in Fig. 1. The lipolytic enzyme has its pH optimum in the range of from about pH 7 to about pH 9, more specifically around pH 8.

Molecular Weight Determination

Mass spectrometry was done using matrix assisted laser desorption ionisation time-of flight (MALDI-TOF) mass spectrometry in a VG Analytical TofSpec. For mass spectrometry, 2 µl of sample obtained according to Example 1 were mixed with 2 µl saturated matrix solution (α-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile (70:30)), and 2 µl of the mixture spotted onto the target plate. Before introduction into the mass spectrometer the solvent was removed by evaporation. The sample was desorbed and ionised by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions were detected by a micro channel plate set at 1850 V. The spectra were calibrated externally with proteins of known mass.

A mass of 28.4 kDa was determined.

N-terminal Amino Acid Sequence

Using standard methods for obtaining and sequencing peptides [*Findlay & Geisow* (Eds.) (1989); Protein sequencing - a practical approach; IRL Press], the following 25 N-terminal amino acid residues of the lipolytic enzyme have

been identified, as presented by SEQ ID NO:1 (where Xaa designates an unknown amino acid residue):

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

Example 3

Lipolytic Activity

Using a monolayer equipment (KSV-5000, KSV Instruments, Finland) it has been demonstrated that the lipolytic enzyme from *Fusarium culmorum* has considerably increased activity towards dicaprin in presence of long chained alcoholethoxylates.

A mixed monolayer in a well defined overall composition, made of a diglyceride substrate and a monocomponent alcoholethoxylate (AEO: Heptaethylene glycol monooctadecyl ether) is spread on an aqueous subphase (10 mM Glycine, pH 10.0, 0.1 mM EDTA, 25°C). The surface pressure is adjusted to the desired value, and a well-defined amount of enzyme (10 LU; lipase units as defined above) is injected into the subphase. Lipolytic action is manifested through the speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolysed into more water soluble reaction products. Using this assay, lipolytic enzymes are discriminated by a parameter θ indicating the final area-fraction of substrate (dicaprin) left *un*hydrolysed by the enzyme as lipolytic activity stops.

In this way, the lipase of the invention was compared to an *Aspergillus* lipase conventionally used in detergents (Lipolase™, available from Novo Nordisk A/S, Denmark). The results are presented in Table 1, below.

Table 1.

Improved tolerance of lipolytic enzyme from *Fusarium culmorum* compared to Lipolase®.

	B (30 mN/m) *
Lipolase®	57%
Fusarium culmorum lipase	25%

* Surface pressure employed.

These results show that when compared to Lipolase®, the lipolytic enzyme obtained from *Fusarium culmorum* is considerably more efficient when alcoholethoxylates are present in the substrate phase.

Example 4

Substrate Affinity

A procedure has been developed aiming at a simple comparison of the ability of lipolytic enzymes to accumulate on/in a substrate phase (olive oil) at alkaline pH (pH 9.0) and presence of the non-ionic surfactant Dobanol 25-7 (2500 ppm) in the aqueous phase.

Procedure

1. Two identical buffer solutions (5 ml) are prepared in 20 ml sealable vials, ("Sample" (s) and "Reference" (r)).
2. Enzyme is added into "Sample" and "Reference" and the lipase concentration is determined (X LU/ml).
3. Olive oil is added onto the "Sample" and both lipase solutions are shaken vigorously. Incubation at 4°C over night.

4. Remaining lipase concentration in the aqueous phases is determined after incubation (Y_i LU/ml; i=r,s).

Summary of incubation conditions

Buffer	100 mM Glycine (5 ml).
pH	9.0.
Substrate	Olive oil (5 ml).
Temperature	4°C.
Lipase activity	5-10 LU/ml.
Incubation	Over night (24-26 hours).

Evaluation of data

The result is calculated by comparing the activity-loss upon incubation in the aqueous phase in contact with olive oil to the activity-loss in the aqueous phase in absence of olive oil:

$\alpha = Y_s/Y_r$ *(see above)*

The results are presented in Table 2, below.

Table 2

Substrate Affinity

Lipolytic Enzyme	α (%)
Lipolase™	99%
Fusarium culmorum lipase	99%

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Fusarium culmorum

(B) STRAIN: CBS 513.94

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:101..1433

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Val Ser Val Ser Thr Thr Asp Phe Gly Asn Phe Lys Phe Tyr Ile
1 5 10 15

Gln His Gly Ala Ala Ala Tyr Xaa Asn
20 20 25

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line s 9-14

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (*including postal code and country*)
Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Nether-
land

Date of deposit	Accession Number
25 October 1994	CBS 513.94

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer A. G. Henriksson
Anne-Grethe Henriksson
Head Clerk

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. A lipolytic enzyme having immunochemical properties identical or partially identical to those of a lipase obtained from the strain *Fusarium culmorum* CBS 513.94.

2. The lipolytic enzyme according to claim 1, which is derived from a strain of *Fusarium culmorum*.

3. The lipolytic enzyme according to either of claims 1-2, which is derived from the strain *Fusarium culmorum* CBS 513.94, or a mutant or a variant thereof.

4. The lipolytic enzyme according to any of claims 1-3, which has a pH optimum in the range of from about 7 to about pH 9, when determined at 30°C with tributyrine as substrate.

5. The lipolytic enzyme according to any of claims 1-4, which has the following N-terminal amino acid sequence:

Ala-Val-Ser-Val-Ser-Thr-Thr-Asp-Phe-Gly-Asn-Phe-Lys-Phe-Tyr-Ile-Gln-His-Gly-Ala-Ala-Ala-Tyr-Xaa-Asn-

6. The lipolytic enzyme according to any of claims 1-5, which has a molecular weight of 28.4 kDa.

7. A process for the preparation of a lipolytic enzyme according to any of claims 1-6, which process comprises cultivation of a lipase producing strain of *Fusarium culmorum* in a suitable nutrient medium, containing carbon and nitrogen sources and other inorganic salts, followed by recovery of the lipolytic enzyme.

8. The process according to claim 7, in which the lipase producing strain is the strain *Fusarium culmorum* CBS 513.94, or a mutant or a variant thereof.

9. A process for the preparation of a lipolytic enzyme according to any of claims 1-6, which process comprises isolating a DNA fragment encoding the lipolytic enzyme; combining the DNA fragment with an appropriate expression signal in an appropriate plasmid vector; introducing the plasmid vector into an appropriate host either as an autonomously replicating plasmid or integrated into the chromosome; cultivating the host organism under conditions leading to expression of the lipolytic enzyme; and recovering of the enzyme from the culture medium.

10. The process according to claim 9, in which the host organism is of bacterial origin, preferably a strain of *Escherichia coli*, or a strain of *Bacillus*, or a strain of *Streptomyces*, or of fungal origin, preferably a strain of *Aspergillus*, a strain of *Neurospora*, a strain of *Fusarium*, or a strain of *Trichoderma*, or a yeast cell, preferably a strain of *Saccharomyces*, or a strain of *Kluyveromyces*, or a strain of *Hansenula*, or a strain of *Pichia*.

11. A detergent composition comprising the lipolytic enzyme according to any of claims 1-6.

12. A detergent composition according to claim 11, which further comprises one or more other enzymes, in particular proteases, amylases, cellulases, oxidases, and/or peroxidases.

13. A detergent additive comprising the lipolytic enzyme according to any of claims 1-6, provided in the form of a granulate, preferably a non-dusting granulate, a liquid, in particular a stabilized liquid, a slurry, or a protected enzyme.

14. A biologically pure culture of the strain *Fusarium culmorum* CBS 513.94.

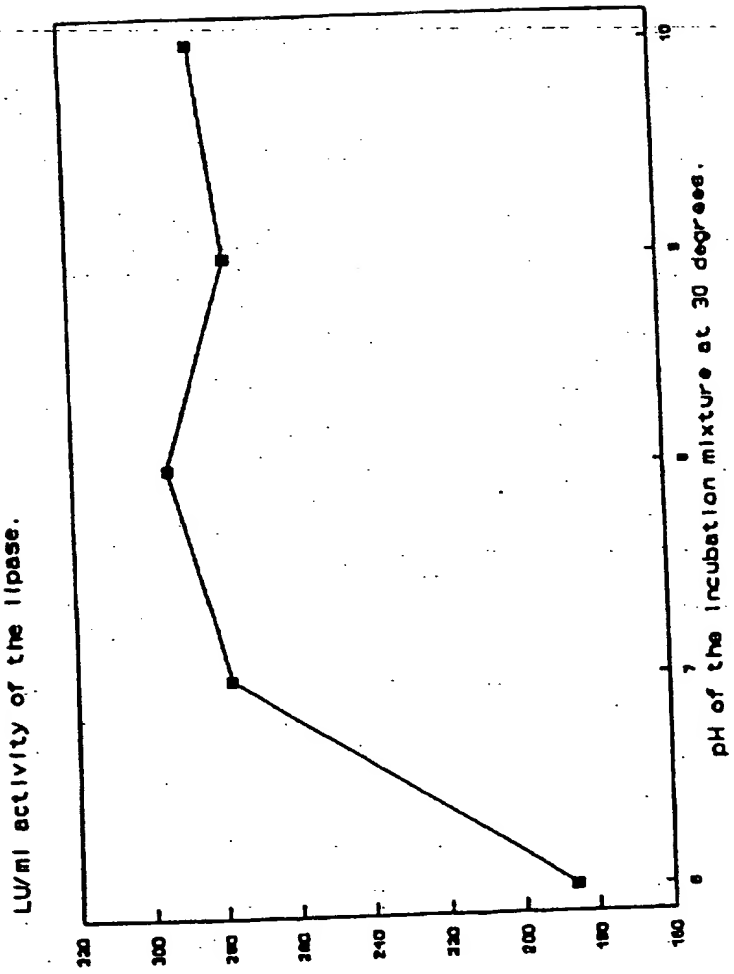
LU/ml activity of the lipase.
pH of the incubation mixture at 30 degrees.
320
300
280
260
240
220
200
180
6
7
8
9
10

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00425

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/20, C12N 1/14
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, US PATENT FULLTEXT DATABASES, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 155, Medline, Dialog accession no. 09225262, Medline accession no. 95155262, Nagao T et al: "Cloning and nucleotide sequence of cDNA encoding a lipase from Fusarium heterosporum"; &J Biochem (Tokyo) (JAPAN) Sep 1994, 116 (3) P536-40	1-14
X	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 4087161, Biosisaccession no. 76037012, GUMENOV V L et al: "The content of cyclic nucleotides and phospho di esterase activity in the ontogenesis of the phyto pathogenic fungus fusarium-culmorum"; & PRIKL BIOKHIM MIKROBIOL 18 (5). 1982. 652-658	1-14

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 February 1996	23 -02- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. + 46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00425

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9403578 A1 (UNILEVER PLC), 17 February 1994 (17.02.94), see page 11, lines 28-32; page 12, lines 4, 31-33; page 13, lines 3-8; page 16, lines 16-32; page 17, lines 30-32; claim 9.	1-14
X	Dialog Information Services, file 5, BIOSIS, Dialog accession no. 3130931, Biosis no. 70080838, LIN T S et al: "Isolation and characterization of a cuticular poly ester cutin hydrolyzing enzyme from phytopathogenic fungi"; & PHYSIOL PLANT PATHOL 17 (1). 1980. 1-16	1-14
X	Dialog Information Services, file 155, Medline, Dialog accession no. 03118570, Medline accession no. 77020570, Soliday CL et al: "Isolation and characterization of a cutinase from Fusarium roseum culmorum and its immunological comparison with cutinases from F. solani pisi"; & Arch BiochemBiophys (UNITED STATES) Sep 1976, 176 (1) p 334-43	1-14
X	EP 0130064 A1 (NOVO INDUSTRI A/S), 2 January 1985 (02.01.85), see the claims	1-14
P,X	US 5439811 A (NOBUHIKO YAMASHITA ET AL), 8 August 1995 (08.08.95), see column 6, lines 58-59	14

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00425

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 3
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

The wording "lipolytic enzyme having immunochemical properties identical or partially identical to those of a lipase..." of claim 1 is not considered to be a technical feature that distinguishes the intended lipolytic enzyme in a clear and concise manner from known lipolytic enzymes obtained from fungi.

It is unlikely that every lipolytic enzyme produced by fungal strains has unique immunochemical properties. Even if such uniqueness exists it has not been shown to be linked to features supporting inventiveness.

The wording "...mutant or variant thereof" of claim 3 is not considered to be clear and concise since the mutant or variant is not restricted to possess the intended, special features of the parent lipolytic enzyme.

Therefore, claims 1 and 3 are not considered to be clear and concise (c.f. PCT Article 6) and the search has been incomplete.

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/02/96

International application No.
PCT/DK 95/00425

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9403578	17/02/94	NONE		
EP-A1- 0130064	02/01/85	AU-B,B-	573225	02/06/88
		CA-A-	1250537	28/02/89
		DE-A-	3473771	06/10/88
		JP-B-	6031414	27/04/94
		JP-A-	60069200	19/04/85
US-A- 5439811	08/08/95	JP-A-	6261756	20/09/94
		JP-A-	7194856	01/08/95

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